AD_____

Award Number: DAMD17-03-1-0530

TITLE: Vitamin E Succinate as an Adjuvant for Dendritic Cell Based Vaccines

PRINCIPAL INVESTIGATOR: Lalitha Ramanathapuram
Emmanuel T. Akporiaye, Ph.D.

CONTRACTING ORGANIZATION: University of Arizona
Tucson, Arizona 85722-3308

REPORT DATE: July 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Annual Summary(1 Jul 2003 - 30 Jun 2004)

4. TITLE AND SUBTITLE
Vitamin E Succinate as an Adjuvant for Dendritic Cell Based Vaccines

5. FUNDING NUMBERS
DAMD17-03-1-0530

6. AUTHOR(S)
Lalitha Ramanathapuram
Emmanuel T. Akporiaye, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Arizona
Tucson, Arizona 85722-3308

E-Mail: lvr@email.arizona.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Dendritic cells (DC) are considered potential candidates for cancer immunotherapy due to their ability to process and present antigens to T cells and stimulate immune responses. However, DC-based vaccines have exhibited minimal effectiveness in abrogating established tumors in mice and human cancer patients. The use of appropriate adjuvants can enhance the efficacy of DC-based cancer vaccines in treating established tumors. In this study we have employed Vitamin E succinate also known as α-tocopheryl succinate (α-TOS), a non-toxic esterified analogue of Vitamin E, as an adjuvant to enhance the effectiveness of DC vaccines in treating established murine mammary (4T1) carcinomas. The hypothesis to be tested is that α-TOS chemotherapy will enhance the efficacy of DC vaccines in treating established tumors and also induce long-term anti-tumor immunity. The rationale for the hypothesis is based on documented evidence that DCs are capable of ingesting apoptotic tumor cells and presenting tumor associated antigens to T lymphocytes to elicit tumor-specific immune responses. The specific aims are to 1) study the effect of α-TOS on tumor cells *in vitro*, 2) determine the efficacy of α-TOS and DC combination therapy in treating pre-established murine mammary tumors *in vivo*, 3) identify the immune mechanism involved in mediating the anti-tumor response.

14. SUBJECT TERMS	15. NUMBER OF PAGES
immunotherapy, dendritic cells, vaccines, vitamin E succinate	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Dendritic cells (DC) are considered attractive candidates for cancer immunotherapy due to their ability to process and present antigens and stimulate the immune system. However DC have not been as effective in treating established disease in animal models. This provides the rationale for combining DC vaccines with a chemotherapeutic drug, which may act as an adjuvant for DC vaccines. Most of the commonly used chemotherapeutic drugs cause tumor cell death but at the same time are toxic to normal cells, which might compromise the ability of the DC to stimulate an effective immune response. Vitamin E succinate or α-TOS is a non-toxic, esterified analogue of Vitamin E that has been shown to be selectively toxic to tumor cell lines *in vitro* as well as inhibit the growth of tumors in animal models *in vivo*. The goal of this study is to enhance the effectiveness of DC vaccines by using it in combination with a non-toxic chemotherapeutic agent, α-TOS. The hypothesis to be tested is that α-TOS will act as an adjuvant for DC vaccines and effectively inhibit the growth of pre-established 4T1 tumors. The specific aims of this study are to 1) study the effect of α-TOS on tumor cells *in vitro*, 2) determine the efficacy of α-TOS and DC combination therapy in treating pre-established murine mammary tumors, 3) identify the immune mechanism involved in mediating the anti-tumor response.

This annual report documents the accomplishments in Aims # 1 and 2 of the proposed study.

RESULTS

1. α-TOS induces killing of 4T1 tumor cells.

In order to demonstrate the susceptibility of 4T1 tumor cells to α-TOS, cells were treated with 40μg/ml α-TOS for 24 hours and clonogenicity and apoptosis assays were performed.

a) For the clonogenicity assay, viable cells obtained after treatment with α-TOS were plated and evaluated for their ability to proliferate and form colonies. The data (Figure 1a) show that, although 50% of the cells were viable after α-TOS treatment only 15% of them had the ability to form colonies as compared with 90% of the ethanol treated (control) cells. This shows that in addition to directly killing tumor cells, α-TOS also suppresses the proliferative potential of surviving cells.

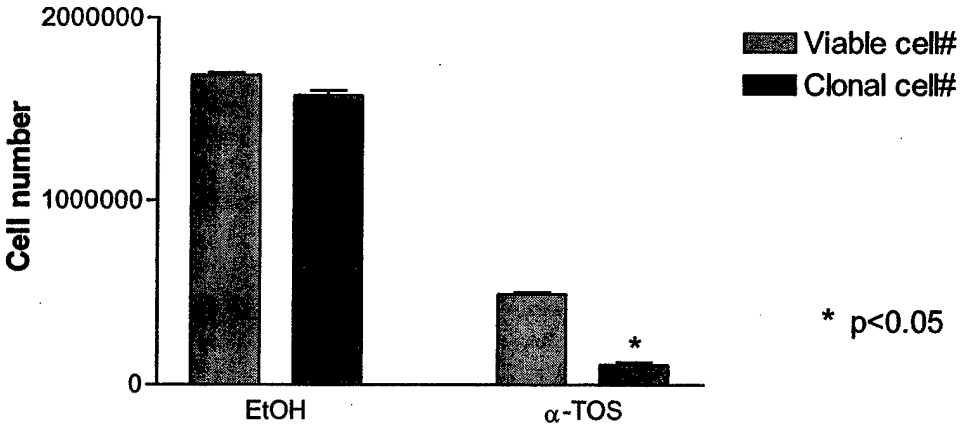

Figure 1a. Effect of α-TOS on clonogenic ability of tumor cells. 4T1 tumor cells were plated at 10^5 cells per well overnight. After 24h the non-adherent and adherent cells were collected and viability assessed using AO/PI. Viable cells were plated out at different dilutions and left undisturbed for 10 days. The clones obtained were fixed with methanol, stained with Giemsa and counted. The graph represents the total number of viable cells compared to the number of clones obtained from those cells.

b) For the apoptosis assay, 4T1 cells were treated with α-TOS, stained with Annexin V/ Propidium iodide (PI) using the Annexin-V-FLUOS staining kit (Roche Applied Sciences) and analyzed by flow cytometry. Annexin binds the phosphatidyl-serine moiety externalized in cells undergoing apoptosis and PI stains dead cells. The data (Figure 1b) show that α-TOS induces apoptosis as a function of time. At 4 hours, 69% of the cells were apoptotic (annexin V positive), which increased to 83% after treatment with α-TOS for 24 hours. In contrast control cells treated with sodium succinate (NaS) did not undergo significant apoptosis.

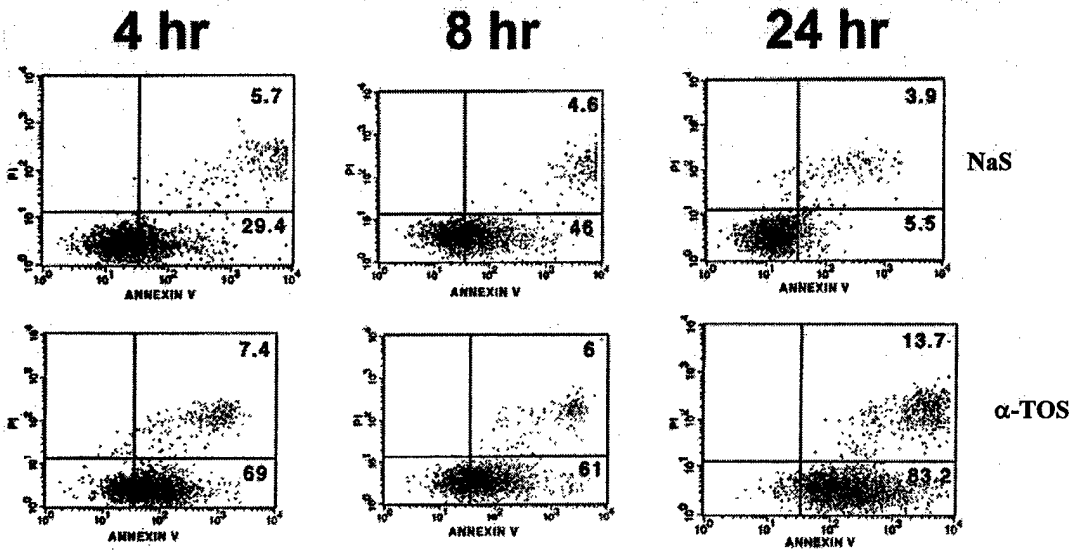

Figure 1b. Apoptosis assay. 4T1cells were plated at 10^5 cells per well in 6-well tissue culture plates overnight. The cells were treated with 40μg/ml α-TOS after 24h. At each time point (4h, 8h, 24h), adherent and non-adherent cells were collected and stained for detection of apoptosis using Annexin V-PI. The numbers in the dot plots represent the percentage of early apoptotic cells (lower right quadrant) and secondary necrotic cells (upper right quadrant) respectively

2. α-TOS is non-toxic to immune cells *in vivo.*

Since α-TOS kills tumor cells *in vitro,* it was important to determine whether it also impaired the functions of immune cells that are required for eliciting an immune response. For the purpose, we injected naïve mice three times with 4mg α-TOS every 4 days. T cells and dendritic cells were isolated from the spleens of the mice 48 hours after the last injection and evaluated for their functional activities. The data (Figure 2) show that α-TOS does not impair the ability of DCs to secrete IL-12 which is a cytokine secreted by mature activated DCs. α-TOS also does not inhibit the proliferation of T cells.

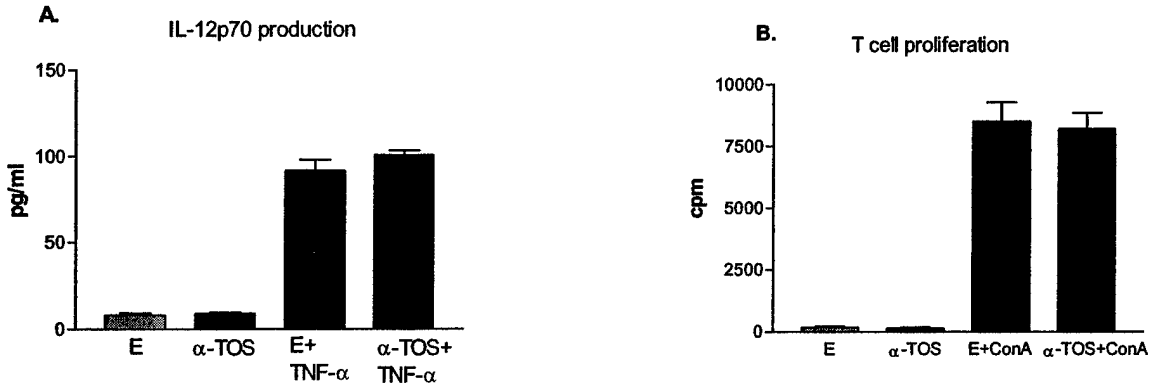

Figure 2. Effect of α-TOS on immune cells *in vivo*. Mice were injected three times with 4mg α-TOS every 4 days. Forty-eight hours after the last injection, splenocytes were isolated from the mice and DCs and T cells were purified using immunomagnetic beads. **A.** $5x10^5$ DCs were set up in triplicate 48-well plates with or without 20ng/ml TNF-a for 24 hours. Supernatants were then collected and assayed for the production of IL-12p70. **B.** $5x10^5$ purified T cells were set up in triplicate in 96-well plates with or without 2mg/ml ConA for 5 days. 1 mCi of [^{3}H]-thymidine was added to each well for the last 18 hours of incubation. Proliferation of T cells was determined by measuring thymidine uptake

3. α-TOS potentiates the anti-tumor activity of DC vaccines on pre-established mammary (4T1) tumors.

We next wanted to evaluate the ability of α-TOS to synergize with DC vaccines in controlling the growth of pre-established 4T1 tumors. For the purpose, mice with palpable 4T1 tumors were injected three times with 4mg of α-TOS every four days (day 14, 18 and 22). The mice were also injected with 10^6 immature DCs on days 16, 20 and 24.Tumor volume was monitored by measuring the tumor using calipers. The data (Figure 3) show that tumor growth was significantly inhibited by a combination of intraperitoneal injection of α-TOS plus subcutaneous injection of DC as compared to α-TOS alone or DC alone. Thus α-TOS alone does not affect tumor growth in an *in vivo* setting but has the ability to synergize with dendritic cells to create an effective anti-tumor response

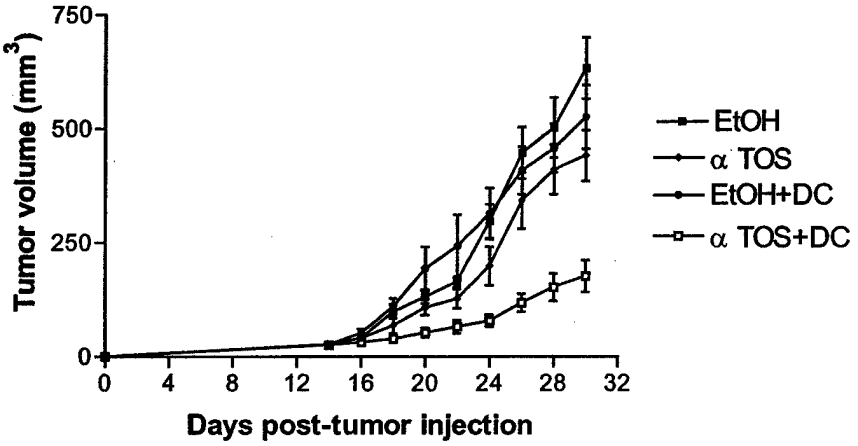

Figure 3. Effect of combined injection of α-TOS and DC on tumor growth *in vivo*. Balb/c mice were injected with $5x10^4$ tumor cells. On development of palpable tumors (day 14), they were injected i.p. with 4mg of α-TOS on days 14, 18 and 22. The mice were injected s.c with 10^6 immature DC on days 16, 20 and 24 and tumor growth was monitored. The figure represents the mean tumor volume± SEM of 7 mice per group

4. Combination therapy with α-TOS + DC induces increased production of IFN-γ by splenic lymphocytes.

Since α-TOS in combination with DCs inhibited the growth of pre-established tumors, we wanted to see if the observed clinical response correlated with elicitation of an enhanced immune response. For the purpose, splenocytes were isolated from mice of each of the treatment groups and re-stimulated with tumor lysate pulsed, TNF-α matured DCs for 48 hours. The supernatants were then collected and assayed for the production of IFN-γ by ELISA. The data (Figure 4) show that splenocytes isolated from mice treated with α-TOS + DC produced significantly higher levels of IFN-γ as compared to cells from mice treated with α-TOS alone or DC alone. This correlates with the tumor growth inhibition seen with the combination treatment.

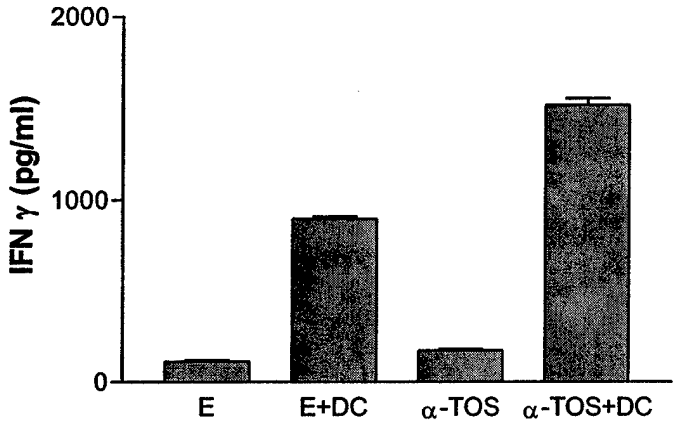

Figure 4. Effect of combined injection of α-TOS and DC on IFN-γ production *in vivo*. Spleens were isolated from 3 mice in each treatment group and pooled. The cells were then re-stimulated with tumor lysate-pulsed, TNF-α matured DC for 48 hours. The supernatants were assayed for the production of IFN-γ by ELISA. The data represent mean ± SEM of triplicate samples.

Key accomplishments

1. **Demonstrate the ability of Vitamin E succinate or Alpha-tocopheryl succinate (α-TOS) to kill 4T1 tumor cells *in vitro***

2. **Demonstrate the non-toxicity α-TOS to immune cells *in vivo***

3. **Demonstrate the ability of α-TOS to act as an adjuvant for dendritic cell (DC) vaccines and inhibit the growth of pre-established 4T1 tumors**

4. **Demonstrate the effect of α-TOS + DC vaccines in inducing the production of IFN γ by splenic lymphocytes *in vivo***

For detailed description of key accomplishments refer to Results section.